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Serum miR-21 and miR-92a as biomarkers in the diagnosis and prognosis of colorectal cancer

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Abstract

Previous studies from our laboratory identified a number of miRNAs that were aberrantly expressed in colorectal cancer (CRC) tissue. However, their diagnostic and prognostic value in serum has not been fully evaluated. In the present study, we measured the levels of 5 miRNAs (miR-21, 31, 92a, 18a and 106a) in serum samples from 200 CRC patients, 50 advanced adenoma patients, and 80 healthy controls by real-time quantitative polymerase chain reaction (RT-PCR). In our study, the levels of miR-21 and miR-92a in patients with CRC and advanced adenoma were significantly higher than those in healthy controls (all $P < 0.05$). MiR-21 yielded an area under the ROC curve (AUC) of 0.802 and miR-92a yielded an AUC of 0.786 in discriminating CRCs from the controls. Additionally, miR-21 and miR-92a yielded an AUC of 0.709 and 0.701, respectively, in discriminating advanced adenomas from the controls. Combined receiver-operating characteristics (ROC) analyses, using both miRNAs, revealed an elevated AUC of 0.847 in discriminating CRCs, and an AUC of 0.722 in discriminating advanced adenomas from the controls. In the multivariate Cox proportional hazards analysis, high miR-92a expression in CRC was independently associated with poor survival ($P = 0.03$; hazard ratio 4.36; 95% confidence interval = 1.64–11.57). In summary, our data indicate that miR-21 and miR-92a serum levels have potential value for early detection of CRC. Furthermore, miR-92a has prognostic value in CRC patients. No significant difference was observed in the levels of miR-18a, 31 and 106a among CRC, advanced adenoma and control samples.

Key words: Colorectal cancer; MicroRNA; Serum; Diagnosis; Prognosis

Abbreviations

miRNA microRNA

CRC colorectal cancer

AUC area under the ROC curve

ROC receiver-operating characteristics

CEA carcinoembryonic antigen

Ct threshold cycle of PCR amplification

Introduction

Colorectal cancer (CRC) is a major cause of cancer-related death worldwide [1], and the incidence and mortality in China have increased rapidly in the past decades [2]. Most CRC-related deaths could be prevented through early diagnosis and surgical removal of early-stage cancer and precancerous lesions. Advanced adenoma, which is an adenoma with significant villous features, a size of 1.0 cm or more and high-grade dysplasia, bridges benign and malignant states in colorectal tumorigenesis, and represents the optimal target lesion for CRC prevention strategies [3]. Several CRC screening tests, including fecal occult-blood test (FOBT) and colonoscopy, are frequently used in detection of CRC. Nevertheless, none of these tests have been established as a well-accepted screening tool due to their invasiveness, high cost or low sensitivity [4]. Thus, there is an urgent need for new noninvasive biomarkers to improve the detection of CRC. In addition, there is a need to identify new, non-invasive prognostic biomarkers for CRC in order to improve postoperative treatment strategies.

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate the translation of specific protein coding genes. Recent studies have revealed the role of miRNAs in a variety of basic biological and pathological processes[5], and the association of miRNA signatures with human diseases has been established [6, 7]. In 2008, Lawrie *et al*. [8] first established the existence of miRNAs in circulation. Soon after, circulating miRNAs have been proposed as sensitive and informative biomarkers in the diagnosis and prognosis of several types of cancer [9-12]. Previous studies from our laboratory identified a number of miRNAs that were aberrantly expressed in CRC tissue [13, 14]; however, their diagnostic and prognostic value

in serum has not been fully evaluated to date.

In this study, we chose 5 miRNAs (miR-21, 31, 92a, 18a and 106a) based on the results obtained from our previous studies [13, 14] and their reported relevance to CRC [15]. Then we examined their expression in serum samples from 200 CRC patients, 50 advanced adenoma patients and 80 healthy volunteers by real-time quantitative polymerase chain reaction (RT-PCR). Furthermore, we investigated the feasibility of using these miRNAs as potential diagnostic and prognostic CRC biomarkers.

Materials and methods

Study cohort

Following ethical approval and written informed consent, whole blood samples were collected from 200 CRC patients, 50 advanced adenoma patients, and 80 healthy age-matched volunteers, which served as controls. All patients were diagnosed and treated at the West China Hospital in Chengdu, China from November 2006 to June 2008. The patient group is representative of the general distribution of patients from West China Hospital. All blood samples were collected one day before surgery, and all advanced adenomas and CRCs were pathologically confirmed. Patients were excluded if they had any of the following issues: clinical diagnosis of familial adenomatous polyposis or hereditary nonpolyposis CRC, diagnosis of other types of cancer at any site at the time of selection, undergoing chemotherapy or radiotherapy before blood sampling, and receiving blood transfusion in past

three months. Information regarding the patients' gender, age, tumor location, size, pTNM stage, differentiation, serum carcinoembryonic antigen (CEA), hepatitis B virus (HBV) carrying and cigarette smoking status was obtained from hospital surgical and pathological records. Tumors were staged according to the UICC tumor-node-metastasis (TNM) staging system. The control blood samples were collected from healthy volunteers with no current or previous malignancy, or inflammatory condition. Clinicopathological features of patients are summarized in Supplementary Table 1.

Follow-up

Follow-up of the patients was performed by a combination of outpatient visits, letters, and telephone calls. Patients were asked to perform physical examinations, blood tests, chest radiography and liver ultrasonography every 3-6 months, and colonoscopy annually. Computed tomography or magnetic resonance imaging was performed when required. For the present analysis, the follow-up data were updated in April 2011. The mean follow-up of patients was 36.4 months (range, 4-53 months).

Sample processing and total RNA extraction

Up to 4 ml of whole blood from each participant was collected in a serum separator tube containing EDTA. Samples were left to clot at room temperature for 30 minutes, and then were centrifuged at 4000 rpm for 10 minutes at 4 °C. Serum was removed, aliquoted and stored at

-80 °C until further use. Total RNA was extracted from 500 µl of serum using the standard TRIZOL LS (Invitrogen, CA, USA) method according to the manufacturer's protocol. Total RNA concentration and integrity were determined with an ultraviolet specrophotometer (Beckman, CA, USA) and a Digital gel image analysis system (Bio-Rad, CA, USA). The median storage time between blood sample processing for serum and endpoint analysis was 32 months.

Selection of serum miRNA markers

A panel of 5 cancer associated miRNAs (miR-21, 31, 92a, 18a and 106a) was chosen based on the results from our laboratory obtained in previous studies [13, 14] and their reported relevance to CRC [15]. MiR-16 was selected as an internal control [9, 16].

MiRNA quantification by real-time quantitative RT-PCR

Total RNA samples were reverse transcribed (RT) to generate specific cDNAs using primers specific to each miRNA target. Specific cDNAs were then amplified by real-time quantitative RT-PCR using TaqMan miRNA primers and probes purchased from Invitrogen. The sequences of primers and probes are summarized in Supplementary Table 2. The protocol used was designed and validated by the others at our laboratory [13, 14]. Briefly, total RNA from 500 µl serum was reverse transcribed with a miRNA RT reaction system and the PCRs were carried out in a final volume of 30 µl using an iCycer iQ System (Bio-Rad). All

reactions, including a no-template control, were run in triplicate. The relative expression of each miRNA was normalized to miR-16 and calculated with the $2^{-\Delta\Delta CT}$ method [17].

Statistical analysis

MiRNA expression levels were compared using the Mann-Whitney U test or Kruskall-Wallis test. Receiver-operating characteristics (ROC) curves were established to evaluate the diagnostic value of serum miRNAs for differentiating tumors from controls. The overall survival was analyzed by log-rank test, and survival curves were plotted according to Kaplan-Meier. Univariate Cox regression was performed on each clinical covariate to examine its influence on patient survival. Final multivariate models were based on step-wise addition. A Wald statistic of P <0.05 was used as the criterion for inclusion in final multivariate models. Data were presented as mean ± SD. All tests were 2-tailed and results with $P < 0.05$ were considered statistically significant. All statistical analyses were performed with SPSS 17.0 software.

Results

Expression of miRNAs in serum samples from patients and healthy controls

Expression of the 5 miRNAs was detectable in all analyzed samples. There were no significant differences in serum miRNA levels with respect to gender and age among CRC patients, advanced adenoma patients and healthy controls (P = 0.114, $\chi 2$ test; P = 0.934, ANOVA). MiR-16 expression in the three groups was not significantly different (P = 0.381), and was therefore used to normalize the RT-PCR data.

To identify the miRNAs that were up-regulated in serum, we first examined the levels of 5 target miRNAs in CRC, advanced adenoma and control samples. Although the miRNAs were previously identified to be relatively abundant in CRC tissues, our results showed that serum miRNA signatures were not fully consistent with that of solid tumors. No significant difference was observed in the levels of miR-18a, 31 and 106a among CRC, advanced adenoma and control samples (P = 0.093 for miR-18a, P = 0.190 for miR-31, and P = 0.214 for miR-106a). Thus, these three miRNAs were not included in further analyses. The expression of the remaining two miRNAs, miR-21 and miR-92a, was significantly elevated in CRC and advanced adenoma samples when compared with controls (all $P < 0.05$, Fig. 1).

Relationship between serum miRNA expression and clinicopathological features

In addition to examining the expression of miRNAs in serum, the relationship between

miR-92a and miR-21 expression and clinicopathological features of patients was examined. The results showed that high miR-92a expression was associated with advanced pTNM stage ($P < 0.001$) and positive nodal status ($P = 0.001$). In addition, high miR-21 expression was associated with advanced pTNM stage (P = 0.045) and poor differentiation ($P < 0.001$). There were no significant associations between miRNAs and gender, age, tumor location, tumor size, CEA, HBV carrying or cigarette smoking (all $P > 0.05$, data not shown).

Expression of miRNAs in serum samples from patients in relation to diagnosis

Since miR-21 and miR-92a levels were significantly elevated in serum of CRC patients in comparison healthy controls, ROC curve analysis was used to explore the potential of using circulating miRNAs as biomarkers for CRC. These analyses revealed that serum levels of both miR-21 and miR-92a were potential biomarkers for differentiating CRC patients from controls with an area under the ROC curve (AUC) of 0.802 [95% confidence interval (CI) = 0.752 - 0.852] and 0.786 (95% CI = 0.728 - 0.845), respectively (Fig. 2A and 2B). At a cut-off value of 0.0043 for miR-21, the sensitivity was 65% and the specificity was 85%. At the cut-off value of 0.00017 for miR-92, the sensitivity was 65.5% and the specificity was 82.5%. Combined ROC analyses resulted in an increased AUC of 0.847 (95% CI = 0.803 – 0.891) with a 68.0% sensitivity and 91.2% specificity (Fig. 2E).

Since the expression of miRNAs in serum of patients with advanced adenomas was higher than in healthy controls, we further investigated the diagnostic value of serum miR-21 and miR-92a expression levels for detection of early lesions in CRC development. ROC

curve analyses revealed that both miRNAs might be helpful to differentiate adenomas from controls, with an AUC of 0.709 for miR-21 (95% CI = 0.618 – 0.801) and 0.901 for miR-92a (95% CI = 0.610 – 0.792) (Fig. 2C and 2D). Combined ROC analyses resulted in an increased AUC value of 0.722 (95% CI = 0.633–0.811) with a sensitivity of 70.0% and specificity of 70.0% (Fig. 2F).

Expression of miRNAs in serum samples from patients in relation to prognosis

Follow-up data was available for 166/200 (83%) CRC patients included in this study. During the follow-up period 38/166 (23%) patients died of CRC. Patients with high serum miR-92a expression had a significantly worse prognosis than patients with low expression, with a 3-year overall survival of 52.2% and 93.8%, respectively ($P < 0.001$, Fig. 3). There was no statistically significant association between miR-21 expression and prognosis ($P = 0.126$).

A Cox proportional hazards analysis was used to further evaluate the potential for serum miR-92a expression as a prognostic biomarker. Univariate survival analyses indicated that miR-92a expression, pTNM stage, differentiation and tumor size were associated with prognosis, while miR-21 expression, gender, age, tumor location, CEA, HBV carrying and cigarette smoking were not. In the multivariate Cox proportional hazards analysis, which included miR-92a, pTNM stage, differentiation and tumor size, high miR-92a expression was independently associated with poor survival ($P = 0.03$; HR = 4.36; 95% CI = 1.64 – 11.57; Table 1).

Discussion

CRC is one of the leading causes of cancer death worldwide. Although great progress has been made in diagnosis and prognosis in the past decades, there is still a need to improve early detection screening methods and to identify new prognostic biomarkers for CRC [1, 2]. Ideal biomarkers should be easy to measure and have a strong association with clinical outcome. miRNAs are could match these proposed criteria [9-12].

In the present study, miR-21 and miR-92a serum levels in patients with CRC and advanced adenoma were significantly higher than those detected in healthy controls. Both miR-21 and miR-92a were potential biomarkers for CRC and yielded an AUC of 0.802 and 0.786, respectively. Combined ROC analyses revealed an increased AUC of 0.847, indicating the additive effect in the diagnostic value of both miRNAs. Furthermore, both miRNAs were helpful in differentiating advanced adenomas from healthy controls with an AUC of 0.709 for miR-21 and 0.701 for miR-92a. Combined ROC analyses revealed an increased AUC value of 0.722, suggesting their potential value for early detection of CRC. In the multivariate Cox proportional analysis, high miR-92a expression was associated with poor survival in CRC patients independent of tumor staging and differentiation.

MiR-92a is part of the miR-17-92 gene cluster, located at chromosome 13q13. As a known oncomir, the miR-17-92 cluster can promote cell proliferation, suppress apoptosis of cancer cells, induce tumor angiogenesis and accelerate tumor progression [18]. Elevated expression of miR-92a has been observed in CRC [10, 12], lung cancer [19] and thyroid cancer [20], suggesting an important role in tumorigenesis. Ng *et al.* [12] reported that

circulating miR-92 was a potential biomarker for CRC diagnosis, which is consistent with our data. Furthermore, our results showed that miR-92a had a prognostic potential, and high miR-92a expression in CRC patients was associated with poor survival. However, contrary results were reported in other diseases. For instance, Tanaka *et al.* [21] showed that miR-92a was dramatically decreased in the plasma of acute leukemia patients using miR-638 as internal control. Shigoka *et al.* [22] demonstrated that the amount of miR-92a in plasma from hepatocellular carcinoma patients was decreased compared with that of healthy donors. These inconsistencies may be due to the different diseases and methods used in different studies.

MiR-21 is expressed at high levels in most solid tumors [23]. Studies in human cell lines showed that miR-21 could target tumor suppressor genes, such as PTEN [24] and TPM1 [25]. Our results showed that serum miR-21 had a diagnostic potential for CRC (AUC = 0.802). We found that miR-21 was also a potential diagnostic biomarker (AUC = 0.709) for advanced adenoma. However, although an association of high miR-21 expression in CRC tissue with poor survival was observed by Schetter *et al*. [6], our results indicated that serum miR-21 expression was not associated with patient survival. These differences may be due to the different samples (serum *versus* solid tissue) or the different quantification methods used. Ng *et al.* [12] demonstrated that miR-21 expression in plasma from CRC patients was lower than in healthy controls, which is inconsistent with our current findings. Nevertheless, the results of Ng *et al.* have to be taken with caution because their study included only five samples.

MiR-18a and miR-106a also belong to the miR-17-92 cluster [18], while miR-31 is located at chromosome 9p21.3 [26], and these miRNAs are expressed at high levels in CRC

tissue [12, 14]. CRC patients with high miR-18a expression tended to have a poorer prognosis than the low expression group [15]. High miR-31 expression in CRC tumor tissue was related to advanced pTNM stage [14]. In our study, no significant difference was observed in serum miR-18a, 31 and 106a levels among the CRC patients, advanced adenomas patients and healthy controls. Our results differ from those of other studies, however this difference may be attributed to the different samples (solid tissue *versus* serum) and methods used.

It has been established that the expression of several miRNAs, including the miR-17-92 cluster [27], miR-31 [28], miR-106a [29], changes in HBV carriers and/or cigarette smokers. China has the largest population of HBV carriers [30] and cigarette smokers [31] in the world. In our study, we examined the possible association of miR-21 and miR-92a expression with HBV status and cigarette smoking in CRC patients; however no association was observed. A possible reason is that the expression of these miRNAs in CRC patients is more influenced by mechanisms involved in tumorigenesis than by HBV infection or cigarette smoking.

One possible concern in this and similar studies of circulating miRNA is the lack of consensus about internal controls for quantitative RT-PCR. Several internal controls have been used thus far in different tumor types, such as miR-16 [9], miR-197 [32], 5S RNA [14] and RNU6B [12]. The results of this study show that miR-16 expression in serum is similar across the patient and control groups, and therefore can be used as a reliable internal control in CRC miRNA expression studies. Secondly, all patients were from the southwestern part of China and most of the patients were relatively poor with high turnover due to work that led to the lack of standard postoperative therapy, regular examination and high-rate follow-up.

Therefore, we were not able to collect enough data to analyze disease-free survival and therapy response in relation to the miRNAs.

In conclusion, miR-21 and miR-92a have potential as non-invasive biomarkers for early detection of CRC. Furthermore, high miR-92a expression is independently associated with poor survival in CRC patients and may be used in the future as a potential prognostic biomarker.

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The National Natural Science Foundation of China was not involved in the study design, collection, analysis and interpretation of data, writing of the report, and decision making to submit the paper for publication.

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Figures Legends

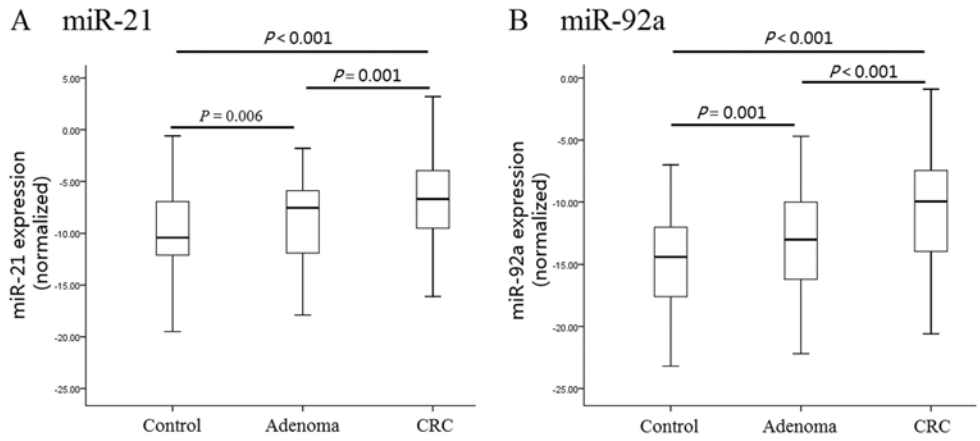

Fig 1. Serum levels of miR-21 (A) and miR-92a (B) from CRC patients, advanced adenoma patients and healthy controls. The lines inside the boxes denote the medians. Expression levels of the miRNAs are normalized to miR-16.

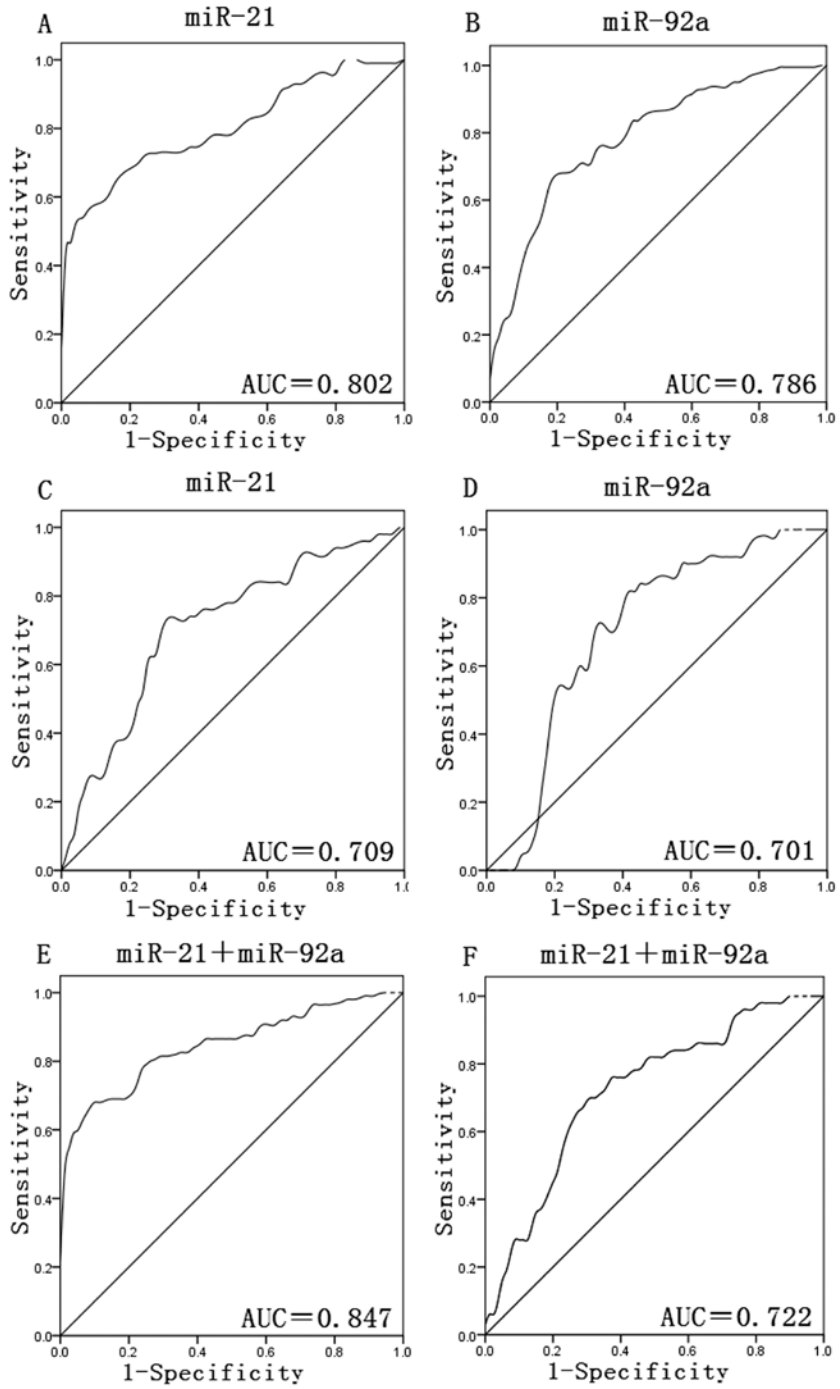

Fig 2. Receiver operating characteristics (ROC) curve analysis using serum miR-21 and miR-92a for discriminating CRCs from healthy controls. (A) ROC curve for miR-21 in discriminating CRCs. (B) ROC curve for miR-92a in discriminating CRCs. (C) ROC curve for miR-21 in discriminating adenomas. (D) ROC curve for miR-92a in discriminating adenomas. (E) Combined ROC curve for miR-21 and miR-92a in discriminating CRCs. (F) Combined ROC curve for miR-21 and miR-92a in discriminating adenomas.

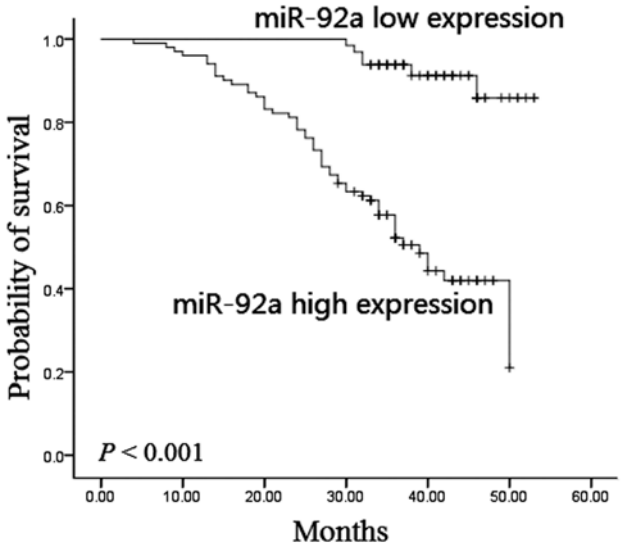

Fig 3. MiR-92a expression in serum in relation to the survival of CRC patients ($P < 0.001$, Log-rank test, Kaplan Meier curve).

Table 1. Univariate and multivariate Cox regression analysis of miR-92a expression levels and prognosis of patients with CRC

Characteristics		Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P* value[a]	HR (95% CI)	*P* value[b]
MiR-92a	Down/Up	10.19(4.05-25.65)	<0.001	4.36(1.64-11.57)	0.03
TNM stage	Ⅰ/Ⅱ/Ⅲ/Ⅳ	3.12(2.23-4.36)	<0.001	2.29(1.58-3.31)	<0.001
Differentiation	Well/Moderately/Poorly/Undifferentiated[c]	3.39(2.47-4.64)	<0.001	2.65(1.90-3.70)	<0.001
MiR-21	Down/Up	1.58(0.77-3.21)	0.126		
Gender	Male/Female	0.96(0.57-1.64)	0.897		
Age (year)	≤50/>50	1.04(0.64-1.79)	0.947		
Cigarette smoking	Negative/Positive	0.97(0.53-1.77)	0.684		
Tumor location	Right/Left/Rectum	0.94(0.68-1.30)	0.935		
Tumor size (cm)	≤5/>5	2.44(1.45-4.12)	<0.001		
CEA (ng/ml)	≤3.4/>3.4	1.38(0.82-2.33)	0.2		
HBV	Negative/Positive	1.39(0.77-2.51)	0.243		

Abbreviation: CRC, colorectal cancer; HR, hazard ratio, CEA, carcinoembryonic antigen; HBV, hepatitis B virus.

[a]Kaplan–Meier method. [b]Multivariate Cox proportional hazard.

[c]Signet ring cell and mucinous adenocarcinoma were included.

Supplementary Table 1. Summary of clinicopathological features of patients used for miRNA analysis

Characteristics		CRCs (n=200)	Advanced adenomas (n=50)	Healthy controls (n=80)
Gender	Male/Female	126/74	25/25	42/38
Age	Mean (Range) (years)	57.09 (20, 89)	57.38 (38, 77)	57.71 (28, 89)
Cigarette smoking	Yes/No	161/39		
Tumor location	Right/ Left/ Rectum	43/37/120		
Tumor size	≤5cm/>5cm	147/53		
pTNM stage	I/II/III/IV	18/96/64/22		
Differentiation	Well/Moderately/ Poorly /undifferentiated[a]	7/134/3/24		
CEA	≤3.4/>3.4(ng/ml)	103/97		
HBV carrying	Negative/Positive	140/60		

Abbreviations: CRC, colorectal cancer; CEA, carcinoembryonic antigen; HBV, hepatitis B virus.

[a] Signet ring cell and mucinous adenocarcinoma were included.

Supplementary Table 2. Sequences of primers and probes for miRNAs

MiRNA		Sequence
MiR-16	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCACTGGATACGACCGCCAATA-3’
	Forward primer	5’-GCGTAGCAGCACGTAAATAT-3’
MiR-18a	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCACTGGATACGCTATCTG-3’
	Forward primer	5’-AAGGTGCATCTAGTGCAGATA-3’
MiR-21	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCACTGGATACGACTCAACATC-3’
	Forward primer	5’-GCTTCGCCTAGCTTATACAGACT-3’
MiR-31	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCACTGGATACGACCAGCTA-3’
	Forward primer	5’-ACGCGGCAAGATGCTGGCA-3’
MiR-92a	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCGCTGGATACGACACAGGCCG-3’
	Forward primer	5’-CCCTGTATTGCACTTGTCC-3’
MiR-106a	RT primer	5’-GTCGTATCCAGTGCTGGGTCCGAGTGATTCGCACTGGATACGCTACCTG-3’
	Forward primer	5’-GCGAAAAGTGCTTACAGTGC-3’
Reverse primer (universal)		5’-CAGTGCTGGGTCCGAGTGA-3’
TaqMan probe (universal)		5’-FAM-CCCGACCCTGCTTAGCTTCCGA-TAMRA-3’